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SECTION FIVE

Attempted Reconstruction of the Pituitary-Adrenal Response to Stress *in Vitro*

MURRAY SAFFRAN
Presented by C. P. LEBLOND, F.R.S.C.

SOME of the rapid metabolic adjustments of an animal organism to the imposition of a change in its internal or external environment (i.e., stress) are mediated by the pituitary-adrenal system. It is difficult to investigate the pathway by which stress activates the pituitary-adrenal system in the intact animal because the manipulation necessary to measure the level of activity of the system constitutes a stress. To overcome this difficulty, we began, in 1951, to reconstruct the pituitary-adrenal system *in vitro*.

The first task was to prove that the rat adrenal gland was able to function and respond to a stimulus *in vitro* in the same manner as the gland *in situ*. In those early days, there were no chemical methods for the measurement of small amounts of adrenocortical hormones, so we turned to a sensitive bioassay procedure. The adrenocortical hormones cause a drop in the number of eosinophilic leukocytes in adrenalectomized mice, and the magnitude of the drop is proportional to the log dose of the hormone (17). Accordingly, rat adrenal glands were incubated for two hours in a Ringer medium under oxygen, and the medium was injected into adrenalectomized mice. The number of eosinophils in the blood fell, indicating the presence of adrenocortical hormones in the medium. Moreover, the addition of corticotrophin (ACTH) to the incubation medium increased the output of adrenocortical hormones by the rat adrenal *in vitro*, as judged by a greater fall of eosinophils (10).

A more rapid method of measuring the production of adrenocortical hormones was developed by utilizing the absorption of ultraviolet light by these hormones. It then became possible to show that the stimulation of the formation of adrenocortical hormones is dependent upon the amount of ACTH added to the medium, and this relationship was suggested as the basis of a bioassay method for ACTH (9).

The *in vitro* study of the adrenal glands led to the discovery of an important aspect of the action of ACTH, which would probably not have been observed *in vivo*. My colleagues, Dr. Birmingham and Dr. Elliott (1) noted that the stimulation by ACTH of the production of adrenocortical hormones sometimes failed to occur. This failure was correlated with the

appearance of a precipitate in the phosphate-buffered Ringer-solution, and was avoided entirely by the substitution of bicarbonate-buffered Ringer medium. The precipitate was calcium phosphate. Further work showed that calcium ions were required for the stimulation by ACTH. We then investigated, one by one, the other components of the medium, and found that glucose is required for the maximal effect of ACTH (16).

We then resumed our development of an *in vitro* bioassay method for ACTH. In the *in vitro* method (12) the adrenal tissue of a single rat is divided into eight parts, which are exposed to both standard and unknown solutions of ACTH, thus decreasing the biological variation. As a result, the *in vitro* assay achieves a satisfactory index of precision with relatively few animals.

Dr. Birmingham and her co-workers continued to study the rat adrenal *in vitro* to identify the kind of adrenocortical hormones formed by the rat (2; 18; 3) and to learn more about the mechanism by which ACTH stimulates the adrenal cortex (2). In collaboration with Dr. Venning and Dr. Giroud, the *in vitro* production of aldosterone was observed and studied (4).

The ACTH that stimulates the activity of the adrenal cortex is formed, stored, and secreted by the anterior lobe of the pituitary gland—the adenohypophysis. With the availability of the bioassay for ACTH, we began to study the adenohypophysis *in vitro*, with the objective of finding the stimulus that results in the release of ACTH from its stores.

The rat adenohypophysis is a small organ, weighing about 5 mg., which can easily be cut into halves. The halved glands were incubated at 37° C in bicarbonate-buffered Ringer solution containing glucose. After one hour, the medium was assayed for ACTH. The tissue released a small amount of ACTH into the medium (11).

The lack of well-defined nerve tracts to the adenohypophysis suggests that the stimulator of the release of ACTH is a blood-borne chemical mediator. There is strong evidence suggesting that the source of the mediator is the neurohypophysial-hypothalamic area of the base of the brain (5). Therefore, bits of tissue from this area were placed in the same container as the halves of rat adenohypophysis, but the release of ACTH did not then differ significantly from the controls, without the brain tissue (11).

The hormones of the adrenal medulla, adrenaline and noradrenaline, are also released by stress, and these substances are also possible mediators of the release of ACTH (6). However, these, by themselves, stimulated the release of ACTH only slightly (11). The possibility remained that the hormones of the adrenal medulla did not act directly on the adenohypophysis, but rather on the base of the brain, to stimulate the release of the mediator. Accordingly, bits of brain tissue, together with a hormone from the adrenal medulla, were added to adenohypophysial halves. The combination resulted in the release of three or more times as much ACTH as was released by the control tissue. The most effective neural tissue was the posterior lobe of the pituitary gland or neurohypophysis (11).

It then appeared that the neurohypophysis, and other areas of the brain, contained a substance or substances which stimulated the release of ACTH. However, the material in the brain is itself only released by substances like those found in the adrenal medulla. Therefore, it might be possible to extract the mediator from various parts of the brain. Indeed, extracts of neurohypophysis and hypothalamus did stimulate the release of ACTH from the adenohypophysis *in vitro* (13).

The neurohypophysis is the site of storage and release of two well-characterized hormones—vasopressin and oxytocin—which influence water metabolism and contract certain smooth muscles. When vasopressin and oxytocin were added to rat adenohypophysis *in vitro*, only the vasopressin seemed to stimulate the release of ACTH. However, when the preparation of vasopressin was purified, the ACTH-releasing activity was found in the "impurities." By repeated chromatography on paper, a material was obtained which stimulated the release of ACTH at a dose of 1 millimicrogram. This material, which was termed a "corticotrophin-releasing factor" (CRF) was distinct from vasopressin, but, on analysis, contained some, but not all, the amino acids of vasopressin, in addition to two amino acids which are not present in vasopressin (15). This preparation also caused a depletion of the ACTH content of the rat pituitary after injection into the living animal (7). In our earlier experiments, vasopressin was thought to be devoid of ACTH-releasing activity (14) but later work revealed that many peptides, including vasopressin, release ACTH, provided that the proper dose is employed (8).

Our current work involves the separation and purification of peptides of various parts of the brain, partly to ascertain which, if any, is the "physiological" CRF, released by stress, and partly to look for peptides which influence the activity of other organs and tissues of the organism.

In summary, our reconstruction of the pituitary-adrenal response to stress *in vitro* has progressed to the point at which the exposure of some parts of the base of the brain to adrenaline or noradrenaline *in vitro* causes the release of material, probably a peptide, which acts on the adenohypophysis *in vitro*, to cause the release of ACTH; the ACTH from the adenohypophysis then stimulates the production of adrenocortical hormones by rat adrenal glands *in vitro*. In the future we shall study in detail the factors affecting the storage and release of the corticotrophin-releasing factor (16) by various parts of the brain, and perhaps by other tissues.

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La Microscopie à fluorescence en microbiologie

JACQUES DE REPENTIGNY
Presented by C. P. LEBLOND, F.R.S.C.

L'INTRODUCTION de nouveaux instruments ou de nouvelles techniques dans l'étude des sciences a souvent été une source de progrès des connaissances fondamentales et des applications pratiques de principes connus. La microbiologie a particulièrement profité de ces apports techniques : ses débuts et les diverses étapes de son développement ont suivi l'apparition et la disponibilité de nouveaux appareils scientifiques.

Grâce aux perfectionnements récents du microscope à fluorescence qui le met à la portée des chercheurs, cet appareil trouve de nombreuses applications en microbiologie. Il est constitué d'un microscope ordinaire muni d'une source de rayons ultra-violets et de filtres sélectifs. De plus, des appareils photométriques comprenant une cellule photoélectrique très sensible peuvent être fixés au microscope pour déterminer quantitativement l'intensité de la fluorescence; l'ensemble constitue un appareil de microfluorométrie.

L'emploi de la microscopie à fluorescence en microbiologie a pris de l'ampleur surtout avec le travail d'Hagemann sur la détection du bacille tuberculeux, en 1937 (7). Par la suite, d'autres auteurs ont appliqué cette technique à la mise en évidence des virus (9), aux « colorations » vitales (20) et à diverses recherches sur les microorganismes (11). En 1953, Goldman (6) a utilisé la technique des anticorps fluorescents (3) dans l'identification des microorganismes. Il a été suivi dans cette voie par de nombreux chercheurs qui reconnaissent la nécessité d'y introduire des éléments de standardisation pour assurer la constance des résultats (2).

Pour notre part, nous avons étudié les aspects fondamentaux de la fluorescence des microorganismes : la fluorescence primaire et la fluorescence secondaire, cette dernière étant conférée par des fluorochromes ou par des anticorps fluorescents. Les résultats de nos travaux ont montré que l'intensité de la fluorescence primaire (autofluorescence) des microorganismes à leur état naturel, n'est pas négligeable. Nous avons toujours retrouvé cette propriété comme une composante assez importante de la fluorescence secondaire. L'observation des couleurs de la fluorescence primaire et la détermination microfluorométrique de son intensité ainsi que celle de la fluorescence secondaire, nous ont permis d'apporter des renseignements objectifs et précis dans le domaine de la cytologie et de la taxonomie

des microorganismes, des analyses de fractions microbiennes et du diagnostic rapide des maladies infectieuses.

Nous nous proposons dans le présent travail d'exposer les résultats de nos travaux sur la fluorescence primaire de même que sur la fluorescence secondaire des microorganismes colorés par des fluorochromes ou par des anticorps fluorescents.

Fluorescence Primaire

Dans le cadre des recherches sur les antigènes de surface, poursuivies par Frappier et ses collaborateurs, nous avons étudié la localisation d'antigènes par les anticorps fluorescents chez certains microorganismes (12; 13). Nous avons remarqué qu'avant d'être traités par des substances fluorescentes, ces derniers avaient une fluorescence primaire assez intense. Sauf quelques observations isolées (8), cette propriété des microorganismes était assez mal connue; il nous sembla opportun d'en faire le sujet d'une étude spéciale, surtout au moment où la microscopie à fluorescence prenait un essor considérable avec l'application de la technique des anticorps fluorescents à l'identification et à l'étude des microorganismes. Nous avons donc recherché et retrouvé l'existence de la fluorescence primaire chez plus d'une trentaine d'espèces examinées (14), en utilisant sur notre microscope un condensateur à fond clair et un objectif sec qui éliminent certaines difficultés inhérentes à l'utilisation du condensateur à fond noir et de l'objectif à immersion.

La généralité du phénomène ayant été vérifiée, nous nous sommes livrés à des études plus détaillées sur ses propriétés. En faisant varier diverses conditions : le milieu et l'âge de la culture, la température et le pH des cellules microbiennes, nous avons observé des différences de couleurs chez les microorganismes étudiées (19). Six espèces ont présenté, en frottis secs sous le microscope, des cellules rougeâtres parmi d'autres qui étaient jaune-verdâtres. Cette fluorescence primaire rougeâtre ne peut être observée que chez les microorganismes séparés du surnageant de la culture et lavés, la culture totale et le surnageant présentant une fluorescence jaune-verdâtre. Ces observations indiquent clairement que chez plusieurs espèces il y a une hétérogénéité de structure chimique d'une cellule microbienne à l'autre au sein d'une population apparemment homogène. Les substances qui provoquent cette fluorescence rougeâtre peuvent être extraites par l'eau, l'alcool et l'acétone. *Sarcina lutea* et *Pseudomonas aeruginosa* ont présenté la plus grande proportion de cellules à fluorescence primaire rougeâtre. *Saccharomyces cerevisiae* ne contenait que quelques cellules rougeâtres. Par contre, l'examen de plusieurs souches de *Staphylococcus aureus* a montré sans équivoque que chez cette espèce toutes les cellules donnent une fluorescence primaire jaune-verdâtre uniquement.

La fluorescence primaire jaune-verdâtre étant de loin la plus répandue chez toutes les espèces étudiées (30 sur 36), nous avons examiné des frottis

microbiens à l'aide du microspectroscope (19) dans l'espoir de retrouver des différences plus fines de couleurs dans la fluorescence primaire de certaines espèces, correspondant à des longueurs d'onde assez précises; mais cette longue étude montra que la fluorescence primaire jaune-verdâtre, une fois décomposée, était constituée de toutes les couleurs du spectre visible. Cependant, celles-ci variaient trop peu en intensité à certaines longueurs d'onde d'une espèce à l'autre, pour permettre une différenciation.

Nous avons alors envisagé une autre possibilité d'investigation : la mesure de l'intensité de la fluorescence primaire de plusieurs espèces bactériennes. La réalisation d'un tel travail supposait la mise au point préalable d'une technique microfluorométrique précise. Nous inspirant du travail d'Ehrlich et Ehrmantraut (4) sur le comptage des microorganismes au moyen de la microfluorométrie, nous avons établi une méthode quantitative (15) pour mesurer sous le microscope l'intensité de la fluorescence primaire de volumes ou de masses microbiennes déterminés. Les spécimens à examiner sont préparés sous forme de frottis secs calibrés sur lame, ou sous forme de suspensions aqueuses d'égale concentration contenues dans des capillaires. La mesure de l'intensité de la fluorescence est effectuée à l'aide d'une cellule photoélectrique très sensible, installée sur l'oculaire de notre microscope à fluorescence. Les lectures se font au moyen d'un amplificateur électronique dont les unités correspondent à 0.0001 de microlumen.

Au moyen de cette technique quantitative d'une très grande sensibilité, nous avons mesuré l'intensité de la fluorescence primaire de plus d'une trentaine d'espèces microbiennes et aussi de plus d'une trentaine de souches d'une même espèce, *S. aureus* (18). Les cultures de tous ces microorganismes ont été faites sur deux milieux différents, l'un à faible et l'autre à forte fluorescence primaire. Les microorganismes ont été séparés des surnageants par centrifugation et lavés à l'eau distillée. Les mesures d'intensité ont été faites sur les microorganismes provenant de deux milieux différents ainsi que sur les deux surnageants de culture correspondants.

Les résultats de ces expériences ont confirmé nos observations antérieures sur l'existence de la fluorescence primaire chez les microorganismes faites à l'œil avant l'utilisation de la technique quantitative. Dans tous les examens que nous avons effectués, nous avons pu mesurer en unités l'intensité de cette propriété des corps microbiens. Celle-ci, lorsque mesurée, se caractérise cependant par une variabilité imprévisible, selon les espèces, les souches, les sous-cultures et les conditions de culture (pH, température, éléments nutritifs, etc.). La variation de l'intensité d'une cellule à l'autre peut devenir assez considérable pour être détectée à l'œil sous le microscope. La microfluorométrie, qui mesure des intensités de fluorescence provoquées par des structures chimiques, met en évidence la variabilité de ces structures soit en qualité soit en nombre au sein des microorganismes. Quant aux surnageants des cultures, ils sont généralement plus fluorescents que les milieux de culture dont ils proviennent.

Les mesures *sur les suspensions aqueuses en capillaires* ont permis de com-

parer les intensités de la fluorescence primaire de microorganismes avec celles de quantités déterminées de certains fluorochromes, ce qui pourrait constituer une base de standardisation de la fluorescence primaire de microorganismes. Au cours de ces examens en *capillaires*, nous avons fait des observations qui à première vue paraissent étonnantes : non seulement les intensités dans le cas de certaines espèces peuvent diminuer ou rester stables pendant une exposition prolongée aux rayons ultraviolets, mais aussi peuvent-elles augmenter dans le cas d'autres espèces (17).

Cette labilité de l'intensité de la fluorescence primaire de certaines espèces de microorganismes pourrait peut-être servir dans certains cas à leur différenciation. Ainsi *Mycobacterium tuberculosis* (souche BCG) subit une hausse d'intensité qui atteint jusqu'à trois fois celle de sa fluorescence primaire, alors que dans le cas de *Candida albicans*, il y a une diminution de trois fois. D'une part, les allures des deux courbes sont nettement opposées et différenciées, et d'autre part l'intensité initiale de *C. albicans* qui est de 5,000 unités plus élevée que celle de *M. tuberculosis* (souche BCG) devient

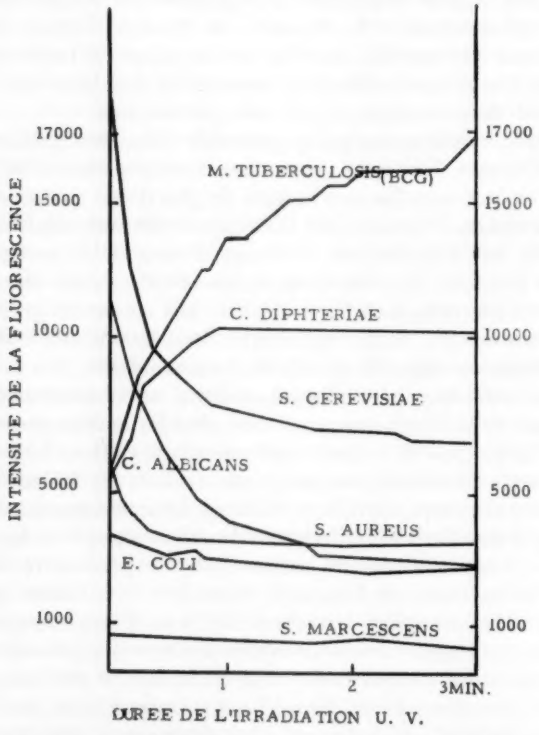

FIGURE 1. Modification de l'intensité de la fluorescence primaire de diverses espèces microbiennes au cours de l'irradiation ultraviolette.

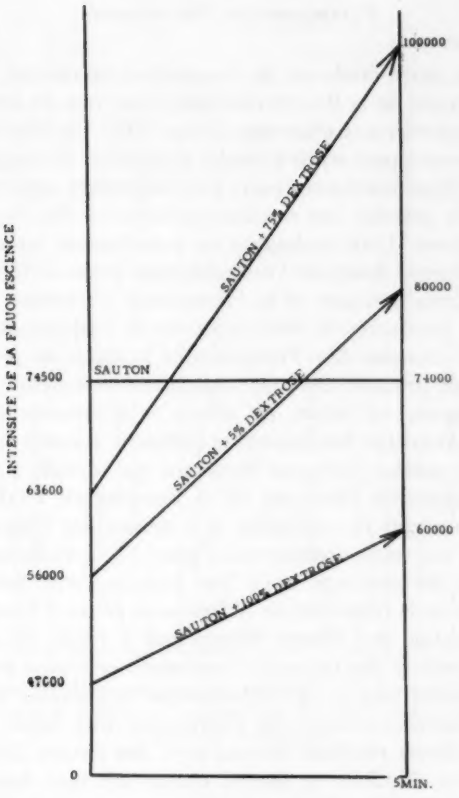

FIGURE 2. Augmentation de l'intensité de la fluorescence primaire des fractions « graisses solubles dans l'acétone » sous l'action des rayons ultraviolets. Ces fractions proviennent de *M. tuberculosis* (souche BCG) cultivés sur le milieu de Sauton et sur des milieux qui sont des modifications de ce dernier.

après trois minutes d'exposition aux rayons ultraviolets, de 15,000 unités plus basse, ce qui fait une différence de 20,000 unités entre les extrêmes (Figure 1). Cette propriété d'augmentation de l'intensité de la fluorescence primaire de *M. tuberculosis* (souche BCG) a pu être retrouvée dans une fraction (les graisses solubles dans l'acétone (5)) de ces microorganismes cultivés sur des milieux qui sont des modifications du milieu de Sauton (Figure 2). Ces résultats suggèrent que les substances qui sont responsables de cette fluorescence dont l'intensité augmente, pourraient éventuellement être isolées et étudiées.

Fluorescence Secondaire

Anticorps Fluorescents

Un aspect de notre étude sur la fluorescence secondaire a porté sur la mesure de l'intensité de la fluorescence spécifique que les anticorps fluorescents peuvent apporter aux microorganismes (16). La détermination objective de cette intensité peut servir à rendre plus précis les diagnostics qui sont faits à l'aide de l'immunofluorescence. Ces diagnostics rapidement effectués, permettraient de prendre des mesures préventives dès l'apparition d'une maladie contagieuse. Cette technique est actuellement mise à l'essai, dans plusieurs laboratoires. Basés sur l'interprétation d'une différence d'intensité entre la fluorescence primaire et la fluorescence secondaire, ces diagnostics sont limités aux possibilités de différenciations de l'œil humain.

Etant dans le moment dans l'impossibilité pratique de prévoir l'intensité de la fluorescence primaire des microorganismes recherchés dans des spécimens pathologiques, au milieu de débris et d'humeurs par eux-mêmes fluorescents, les anticorps fluorescents se prêtaient mieux à une standardisation. Nous avons mis au point une technique qui consiste à évaluer à l'aide de la microfluorométrie l'intensité de la fluorescence totale des solutions d'anticorps fluorescents en capillaires et à déterminer l'intensité spécifique qu'ils apportent aux microorganismes (Figure 3); le maintien de la qualité de la spécificité des anticorps après leur couplage aux fluorochromes est vérifié au moyen de la technique de diffusion en gélose d'Ouchterlony.

La standardisation des sérums fluorescents à l'aide de ces techniques, éviterait de poursuivre des travaux d'immunofluorescence avec des réactifs douteux. Elle permettrait : (a) l'élimination des sérums fluorescents qui confèrent aux microorganismes une fluorescence trop faible; (b) des comparaisons entre divers résultats obtenus avec des sérums différents; (c) la détermination des dilutions de sérums encore efficaces dans le diagnostic (16).

Selon nos déterminations quantitatives, l'intensité de la fluorescence primaire représente jusqu'à la moitié de celle de la fluorescence totale qui est observée après le traitement des microorganismes par les anticorps fluorescents.

Fluorochromes

Microfluorométrie. Les microorganismes traités par des fluorochromes ont été étudiés au moyen de notre méthode microfluorométrique, sous forme de suspensions aqueuses en capillaires et aussi de frottis secs.

En capillaires, la labilité caractéristique de l'intensité de la fluorescence primaire de certains microorganismes peut être retrouvée même après leur fluorochromisation à l'aide de quatre fluorochromes différents, tous solubles dans l'eau : la fluorescéine Na, la rhodamine B, l'auramine O et l'orangé d'acridine. Nous avons ainsi étudié une dizaine d'espèces microbiennes.

Cependant, l'intensité de la fluorescence primaire de *M. tuberculosis* (souche BCG) a présenté un comportement particulier surtout au cours de

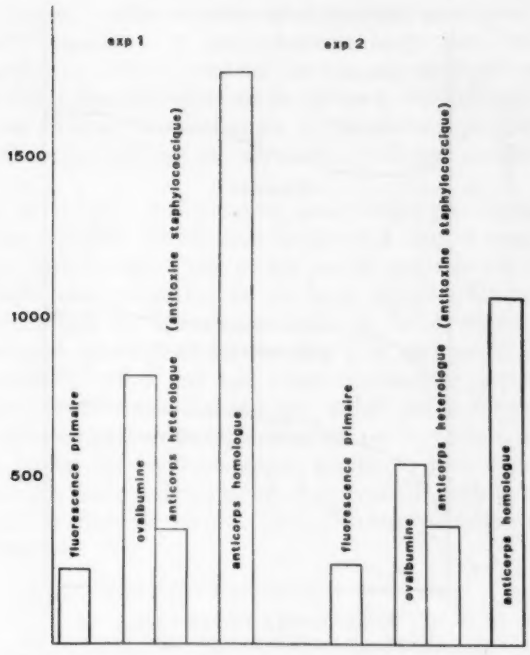

FIGURE 3. Détermination de l'intensité de la fluorescence secondaire spécifique conférée aux microorganismes par les anticorps fluorescents.

sa coloration par l'auramine O selon le procédé d'Hagemann, employé dans le diagnostic de la tuberculose (7). L'auramine O ajoute sa propre intensité, qui avant traitement par l'acide chlorhydrique masque l'augmentation de la fluorescence primaire dans les premières secondes de l'irradiation ultraviolette. Après le traitement par l'acide chlorhydrique, l'intensité ajoutée par l'auramine O ne modifie plus l'allure de la courbe montante de la fluorescence primaire (Figure 4).

Nous avons aussi mesuré sur de frottis secs, l'intensité da la fluorescence secondaire qui est conférée aux microorganismes par les fluorochromes. Celle-ci est obtenue en soustrayant l'intensité de la fluorescence primaire de celle de la fluorescence totale. Au cours de ces mesures, nous avons fait des observations inattendues : ainsi l'auramine O, qui en solution ne fluoresce que très peu, confère aux microorganismes une fluorescence extrêmement intense. Ce phénomène laisse supposer qu'il se produit par interaction physicochimique, entre les substances présentes dans les microorganismes et l'auramine O, des complexes ayant une fluorescence très élevée.

Il est à remarquer que pour une même culture, l'emploi d'une concentra-

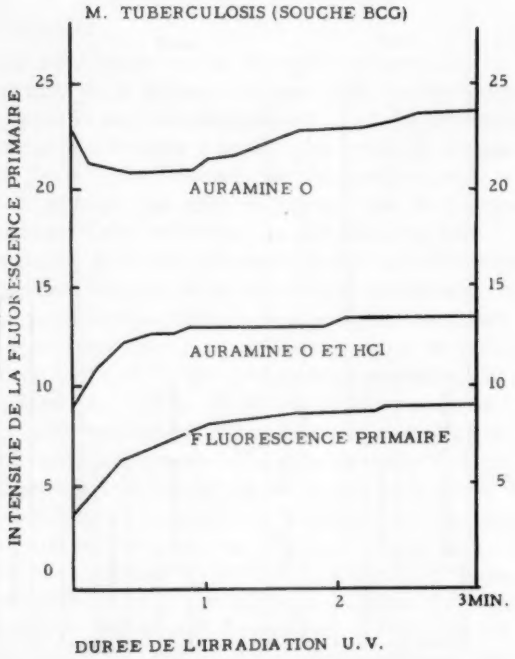

FIGURE 4. Etude microfluorométrique de la coloration à l'auramine O pour acido-résistants. Modification de l'intensité sous l'action des rayons ultraviolets à diverses étapes du procédé.

tion dix fois plus faible de fluorochromes ne correspond pas nécessairement à une diminution équivalente d'intensité de la fluorescence. De plus, le rapport entre les intensités ajoutées par les divers fluorochromes se modifie d'une espèce à l'autre, ce qui pourrait aider à leur identification.

L'introduction d'une méthode quantitative a permis de mettre en lumière des aspects nouveaux de la fluorescence secondaire des microorganismes, qui augmentent nos possibilités d'investigation chez ces derniers.

Même si l'intensité de la fluorescence secondaire des microorganismes traités par diverses dilutions de fluorochromes, est généralement plus élevée que celle de leur fluorescence primaire, elle peut aussi en certains cas être plus faible que cette dernière.

Etude des acides nucléiques microbiens. Plus récemment, nous avons entrepris une étude sur les relations qui pourraient exister entre les acides nucléiques et la composition antigénique du staphylocoque.

Armstrong (1) avait signalé en 1956 la possibilité de déceler au microscope à fluorescence les acides nucléiques chez *Escherichia coli*, au moyen de

l'orangé d'acridine, ce fluorochrome donnant une coloration verte avec l'acide désoxyribonucléique et une coloration rouge avec l'acide ribonucléique. Marston en 1961 a combiné l'action des nucléases avec celle de l'orangé d'acridine dans une étude sur les formes L des staphylocoques (10).

Dans le cas présent, la microscopie à fluorescence permet l'étude de macromolécules microbiennes de caractère génétique comme les acides nucléiques.

Au cours de travaux préliminaires, nous avons pu obtenir avec des staphylocoques typiques, des résultats similaires à ceux d'Armstrong et de Marston. Les staphylocoques non traités par les enzymes spécifiques, donnent une fluorescence rougeâtre, ce qui laisse supposer un prédominance d'acide ribonucléique. La détection exclusive de l'acide ribonucléique chez les staphylocoques intacts pourrait être due à la destruction préalable de l'acide désoxyribonucléique par une désoxyribonucléase présente chez les microorganismes eux-mêmes. Les témoins, traités par la ribonucléase, présentent une fluorescence verdâtre, produite soit par la réaction entre l'orangé d'acridine et l'acide désoxyribonucléique, soit par la fluorescence primaire.

Ces expériences sont encore en cours, et les résultats obtenus au moyen de la microscopie à fluorescence sont actuellement contrôlés par diverses méthodes d'analyse.

Résumé et Conclusion

Les résultats de nos travaux suggèrent que la microscopie à fluorescence et la microfluorométrie pourraient contribuer au progrès des recherches dans plusieurs domaines de la microbiologie : (1) Les diverses couleurs de la fluorescence primaire pourraient constituer de nouveaux critères de différenciation entre des espèces, des souches et même des cellules d'une même culture pure. (2) Les différences d'intensité de la fluorescence primaire pourraient servir à distinguer certaines espèces et même certaines fractions microbiennes, parce que la microfluorométrie met en évidence des différences de structures chimiques. De plus, les modifications que subit l'intensité de la fluorescence sous l'action prolongée des rayons ultraviolets semblent caractéristiques pour plusieurs espèces. (3) La détermination de l'intensité de la fluorescence de microorganismes traités par des anticorps fluorescents et de celle de témoins non traités, permet d'évaluer une différence d'intensité qui est due au pouvoir « colorant » spécifique d'un sérum fluorescent : cette mesure objective pourrait contribuer à la standardisation des techniques d'immunofluorescence. (4) Les différences entre les intensités conférées aux microorganismes par divers fluorochromes pourraient constituer des critères utilisables dans la caractérisation des espèces. (5) La spécificité des couleurs de la fluorescence des complexes formés par les acides nucléiques microbiens et l'orangé d'acridine, semble assez sûre pour constituer une méthode d'étude de la génétique microbienne et des problèmes connexes.

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TRANSACTIONS OF THE ROYAL SOCIETY OF CANADA
VOLUME LV : SERIES III : JUNE, 1961
SECTION FIVE

Amino Acids and Activities of Brain

K. A. C. ELLIOTT
Presented by C. P. LEBLOND, F.R.S.C.

IN the neurochemistry laboratory at the Montreal Neurological Institute we have interested ourselves in the energy-yielding metabolism of brain, in the rôle of substances that, like acetylcholine, may be concerned directly in physiological function, in the interrelations of electrolytes with metabolism, and in the pathological condition of cerebral oedema. It would be expected that no aspect of the activity of a tissue would be completely separate from other aspects and, in the field to be discussed, interconnections between all the aspects just mentioned, and several others have become specially apparent.

The dicarboxylic amino acid, glutamic acid, has long been of special interest to brain chemists since early work by Quastel and Weil-Malherbe. They found that glutamate was the only amino acid tested which would support oxygen uptake by brain tissue and that glutamic acid or its amide, glutamine, exerts marked effects on glycolysis, the initial splitting of glucose which yields lactic acid. These effects were shown to be specific for brain tissue.

Krebs and his co-workers showed that brain tissue contains large amounts of glutamic acid and glutamine and that the tissue will actively absorb glutamate from the surrounding fluid provided that oxygen and glucose or another suitable substrate are provided.

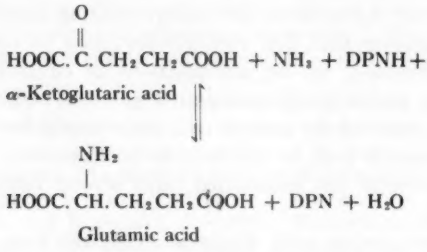
Further interest in glutamate arose from the studies of Waelsch and others who observed apparent improvement in certain *petit mal* epileptics and mentally defective children when they were fed large amounts of glutamate. As has been the case with so many other forms of treatment, the value of glutamate feeding seemed to disappear after the first few trials. There remains, however, the suspicion that glutamate may be concerned with mental conditions. Glutamate in the plasma does not pass the barrier between blood and brain in sufficient amount to affect the brain content but glutamine does, and perhaps some glutamate is converted in other organs to glutamine and can enter the brain in this form. Glutamate is also of general interest as the substance that gives the flavour to soya bean and other sauces and soups.

The development of chromatographic methods led to the discovery that brain contains, in free form, quite large amounts of several other amino acids. The most interesting of these was γ-aminobutyric acid (GABA). This

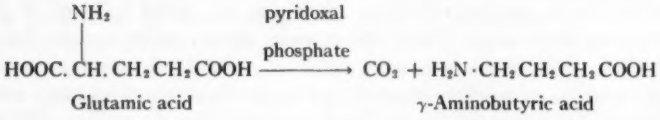
substance had been found to be present or formed in a variety of micro-organisms and plants but it had never before been detected in mammalian tissue and it was found to be present only in brain.

This work and studies on the metabolism of GABA were carried out mainly by Eugene Roberts and J. Awapara and their co-workers beginning in 1950.

Glutamic acid is derived by enzyme-catalysed reductive amination from α-ketoglutarate acid. α-Ketoglutarate is an intermediate in the Krebs metabolic cycle through which most residues of carbohydrate, fat, and protein are catabolized to carbon dioxide and water in all tissues.

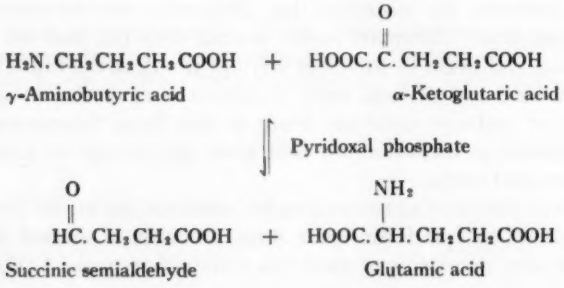


GABA is formed from glutamate by decarboxylation caused by a decarboxylase.



In the mammal this enzyme is found only in brain. It is dependent for its activity on a coenzyme, pyridoxal phosphate. Pyridoxal is one of the B group of vitamins, vitamin B_6. The coenzyme is relatively loosely bound to the decarboxylase apoenzyme.

GABA is metabolized further by reaction with α-ketoglutarate under the action of a transaminase, yielding succinic semialdehyde.



This enzyme is also dependent on pyridoxal phosphate which is more firmly bound to the apoenzyme in this case.

Succinic semialdehyde is oxidized by a pyridine nucleotide-dependent dehydrogenase to succinic acid.

$$H_2O + HC(=O).CH_2.CH_2.COOH + DPN \rightleftharpoons HOOC.CH_2.CH_2.COOH + DPNH + H^+$$

Succinic semialdehyde — Succinic acid

As a result of the presence of these four enzymes in brain, the carbon atoms of glucose, the main substrate of brain metabolism, can become the carbon of glutamate and GABA and this GABA pathway provides an alternative route for the metabolism of glucose in brain, as is shown in Figure 1.

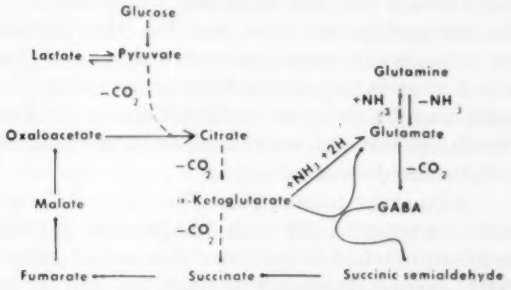


FIGURE 1. Interrelations of the glutamate-GABA system with the citric acid cycle. Dashed lines indicate that steps are omitted. Relations with other amino acids, such as alanine from pyruvate and aspartic acid from oxaloacetate, are not shown.

The fact that carbon from glucose is partly metabolized via the GABA shunt has been amply proved by isotope methods.

While these metabolic studies were being made, several other quite different types of studies were going on, all of which were to converge into a single theme.

In 1953 Ernst Florey discovered that extracts of brain would produce excitatory and inhibitory effects when applied to the stretch receptor organs of the crayfish. These organs consist of pairs of fine muscle fibres in which the dendrites of two sensory nerve cells are embedded. The axons of these cells lead off into a nerve. The organ and the nerve supplying it can be dissected free of other structures and the nerve may be attached to an electrode which leads to an amplifier and loud speaker. One of the nerve cells is found to fire briefly when strong stretch is applied. The other, the "slow-adapting" neuron, will fire steadily when constant stretch is applied to the receptor.

The presence of a possible inhibitory transmitter substance in brain and a method for testing its activity were of great neurophysiological interest, and Dr. Florey joined me at the Neurological Institute in 1955 to study the inhibitory factor, which he called "Factor I." The excitatory activity observed earlier seemed to be a non-specific effect which rarely occurred under controlled conditions. But the inhibitory effect was clear. Brief application of a buffered saline extract of brain causes the slow-adapting neuron to cease firing for a period that depends on the strength of the extract. The activity of the neuron returns immediately after the inhibitory solution is washed off. The interest of these brain extracts to mammalian physiology was established by Dr. Florey and Dr. McLennan who used a partially purified preparation and showed that it had inhibitory effects when applied to mammalian ganglia and spinal cord.

We developed the stretch receptor test into a remarkably accurate quantitative assay for Factor I. We were then able to interest Merck and Co. of New Jersey in our problem and they sent Dr. Alva Bazemore, who was experienced in isolation procedures, to work with us. Starting with 100 lbs of beef brain and using rather standard methods of solvent extraction and chromatography, and following the fractionations of the Factor by means of our assay, crystalline material was isolated and was identified with GABA. Authentic GABA showed the same activity.

At the same session of the International Physiological Congress in Brussels in 1956 at which we reported this work, the Japanese physiologist Hayashi reported experiments in which it was found that several omega-amino acids, including GABA, exerted depressant effects when injected in rather large amounts into the cerebral ventricles or carotid arteries of dogs. In Hayashi's experiments the most active compound tested was γ-amino-β-hydroxybutyric acid. We find this substance about half as active as GABA on the stretch receptor and, though Japanese workers have reported its presence in brain, we have not been able to detect it.

We and others have found several other substances, including β-alanine and γ-amino-α-hydroxybutyric acid, to have some Factor I activity. But work by Dr. Levin and Mr. Lovell in our laboratory has shown that GABA, determined by chromatographic-chemical and enzymatic methods, can account for all the Factor I activity as ordinarily determined in various regions of brain. The proportion of the total activity which is contributed by any other active substance is negligible.

However, chromatographic studies by McLennan indicated the presence of another active substance in considerable amounts. We believe we have identified this as glutamic acid which is present in large amounts in brain and which we find exerts variable effects, inhibitory or excitatory. We normally eliminate its interference in the assay by placing excess of glutamate in all solutions. But a receptor organ that is not adapted to the presence of glutamate can be inhibited by this substance, especially if the solution is unbuffered and consequently slightly acid. We believe that the effects of

glutamate, varying with conditions, may be of considerable neurophysiological significance.

At the international conference in Brussels already mentioned another aspect of the story was reported by Pfeiffer on behalf of his colleagues Killam and Bain. Convulsions were induced in rats by the administration of semicarbazide, thiosemicarbazide, or related substances. In the brains of these animals it was found that GABA, alone among the free amino acids, was decreased in amount. The carbazides are aldehyde-trapping agents and it was shown that they inhibit the production of GABA from glutamic acid by immobilizing the loosely bound aldehydic coenzyme, pyridoxal phosphate. At about the same time further interest was aroused as a result of an emergency in which a certain baby food had to be withdrawn from the market because its use resulted in convulsions. It was found that the convulsions were due to a deficiency of vitamin B_6, pyridoxal, in the baby food. Soon after this, vitamin B_6 deficiencies due to several other conditions were identified as the cause of convulsions in infants. Isoniazid, used in treating tuberculosis, is a hydrazide and can cause convulsions as can a number of pyridoxal analogues. It seems likely that all these effects are due to interference with GABA production but we have to remember that pyridoxal phosphate is concerned in a large number of other metabolic processes and the relation to GABA may not be the whole or even the main story.

Immediately after the identification of GABA with a neuro-inhibitory factor there was an outburst of work on the effects of GABA and related substances by biochemists, pharmacologists, and particularly by neurophysiologists. Topical application of GABA solutions was found to exert marked effects on the electrical activity of the brain. An example of such effects appears when the electrical response of the cerebral cortex to adjacent electrical stimulation is recorded. As Figure 2 shows, the polarity of the response is reversed by application of GABA solution to the surface of the brain.

The actual rôle of GABA as a neurotropic agent is not clear. At first we thought it might be a transmitter substance for inhibitory impulses. Studies by Kuffler and Edwards indicate that this could be true in the crustacean. Purpura and Grundfest maintain that, when applied to the mammalian brain, GABA and related substances do not act on inhibitory synapses but block excitatory synapses. It is not obvious what physiological function such blocking would normally serve.

The well-known transmitter substance, acetylcholine, is extremely rapidly destroyed by acetylcholinesterase after it has been liberated and has exerted its action. But van Gelder and I found that brain tissue does not cause net destruction of GABA. GABA is metabolized and replaced with no net change in concentration. If brain slices are provided with oxygen and glucose, they will absorb large amounts of GABA from solution in the surrounding medium. The endogenous GABA, and much of that absorbed,

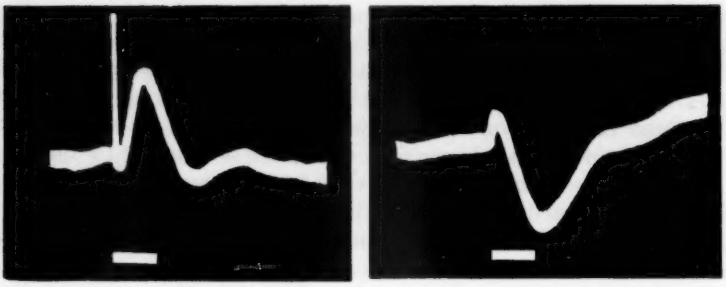


FIGURE 2. Effect of topical application of GABA on the direct cortical response to electrical stimulation in the cat. Left: surface-negative response before GABA. Right: surface-positive response to the same stimulus almost immediately after applying 1 per cent GABA in Ringer solution. Bars indicate 10 milliseconds. (Typical records obtained by Dr. Salvador Gonzales, Montreal Neurological Institute.)

is found to be present in an occluded state in which it is inactive on the stretch receptor test object. The occluded GABA is readily released by heat, hypotonic fluid, or mild acid or alkali.

Van Gelder and I found that the amount and state of GABA (determined by Factor I assay) are not affected by most neurotropic drugs other than those which are known to interfere with pyridoxal phosphate. We found that the anti-depressant drug, iproniazid, elevates the GABA content of the brain, the *free* material being mostly affected. The *decrease* in GABA caused by the convulsant carbazides seems to affect mainly the *occluded* GABA. If both thiosemicarbazide and iproniazid are administered, the total GABA in the brain, occluded plus free, may be in the normal range yet convulsions still occur. We tentatively conclude that the effective GABA is the occluded material, the level of which is lowered by the convulsant carbazides, while the increase in free GABA caused by iproniazid is without obvious effect. When GABA is applied topically to the brain *in vivo* its effects cannot usually be immediately reversed by rinsing; the effects disappear only slowly. This also suggests that it is not free GABA but GABA bound in the tissue that is effective. I believe, therefore, that GABA in brain is not a transmitter substance but is absorbed on certain cellular membranes and in this condition moderates neuronal excitability.

In the crustacean, but not in the mammal, GABA exerts neuromuscular effects. If GABA is injected into the haemocoele of a crayfish, the animal goes completely limp. If the parent substance, glutamate, is injected, the animal goes into a rigid tonic condition. Thus these two substances, differing only by a carboxyl group, can exert opposite effects. Curtis and Watkins in Canberra have shown that such opposite effects occur also when glutamate and similar dicarboxylic acids and GABA and other omega-amino acids are brought into contact with spinal neurons of the cat. However, as I noted

earlier, glutamate can behave *like* GABA under some conditions. There are thus several possibilities whereby metabolic changes in the brain can affect excitability through changes in the amounts, state, and type of activity of these amino acids.

There are other ways in which these amino acids affect brain activity. It is well known that ammonia is released in neuronal activity and it is known that ammonium ion can exert toxic or convulsant effects. The formation of glutamic acid from α-ketoglutarate involves uptake of ammonia and so does the further conversion of glutamic acid to glutamine. There is considerable evidence that the rôle of this amino acid system as an ammonia buffer is important in the activity of the brain. During convulsions induced in normal brain by drugs or electrical stimulation, the ammonia and the glutamine content of the brain increases. Dr. Quastel's group finds that the presence of certain convulsants increases glutamine formation *in vitro* and a narcotic decreases it. Berl, Purpura, Girado, and Waelsch find that in local lesions produced by freezing the glutamine content decreases as seizure activity develops. Perhaps these lesions become epileptic foci because their capacity to remove ammonia by glutamine formation is diminished.

Several authors, including particularly Dr. Quastel and his co-workers, have shown that brain tissue absorbs a variety of amino acids from the surrounding medium against the concentration gradient, provided oxygen and glucose are available. To some extent these amino acids become incorporated into proteins. Others have shown that, *in vivo*, there is considerable breakdown and re-synthesis of certain proteins and the rate of these is related to physiological activity. Our studies on the occult form of GABA led us to investigate other amino acids and my co-worker Mr. Fernand Bilodeau has recently shown that all the amino acids that are present in brain in large amounts are partially in an occult form from which they are readily released, though none is occluded in such high proportion as GABA.

Changes in the distribution of electrolytes across membranes are of course of fundamental interest in cellular and particularly nerve physiology and amino acids seem to be intimately concerned in the distribution of electrolytes in brain tissue. Krebs and his co-workers showed that the uptake of glutamate by brain slices is accompanied by increased uptake of potassium. Conversely Dr. Quastel and his co-workers have shown that, in the presence of a high potassium concentration in the bathing medium, the rates of production of glutamate, glutamine, and GABA from glucose are increased. This suggests an accentuation of this route of metabolism when the stimulus of potassium is applied.

A peculiar characteristic of brain tissue is its tendency to take up fluid and swell. As cerebral oedema, causing increased intracranial pressure, this tendency gravely accentuates the effects of head injuries and poses serious problems in neurosurgery. We find that two kinds of swelling, apparently intracellular and extracellular, can be observed with brain *in vitro* and that

the former is strongly increased by the presence of glutamate, or of high potassium, in the bathing fluid.

In early work on acetylcholine synthesis by brain extracts it was found that the system was accelerated by glutamate. This action has not been explained but it suggests yet another rôle of specific amino acids in brain.

I should mention that amino acids other than those with which I am particularly concerned are closely related to the citric acid cycle. Others seem to have extremely interesting rôles as parent substances of very active substances present in brain. I refer to phenylalanine and tyrosine which are converted to adrenalin, noradrenaline, and dopamine and to tryptophan which yields serotonin, and perhaps histidine which yields histamine.

Thus we find that certain amino acids occur in considerable concentrations in brain and are in a pathway of intermediary metabolism which is peculiar to brain and, in this tissue, this pathway constitutes a quantitatively significant route for the carbon of glucose and other substrates. On the other hand, glutamate and glutamine exert strong effects on the earlier glycolytic phase of glucose metabolism. The glutamate pathway, and its glutamine side issue, are concerned in the disposal of the ammonia produced in nervous activity. This pathway also involves the intermediate formation of GABA and glutamate which have profound effects on the physiological function of nervous structures. These amino acids affect the potassium-sodium distribution which is intimately concerned in function and, reciprocally, extracellular potassium ion concentration affects the distribution of these amino acids. Glutamate is concerned in one form of tissue oedema and has been associated with epilepsy and mental deficiency.

We thus have a kind of biochemical jigsaw puzzle—a set of extremely varied but apparently interrelated observations. I believe we will not be able to fit the parts of this puzzle together to give a full picture until we know the appropriate "table" to lay it out on. By this I mean the structure with which all the pieces interact. My idea of this table or structure is some cellular membrane, probably a lipo-glyco-protein structure, which is concerned in nervous function and which is modified in various ways according to the way certain amino acids occupy certain receptor sites on it. I think the observation that "free" amino acids in the brain are not entirely free but are to some extent present in an occult or adsorbed form may be a step towards understanding the substrate and obtaining a more complete biological picture.

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TRANSACTIONS OF THE ROYAL SOCIETY OF CANADA
VOLUME LV : SERIES III : JUNE, 1961
SECTION FIVE

Protective Mechanisms against Nerve Poisons in Insects

A. W. A. BROWN, F.R.S.C.

THE central nervous system of insects consists essentially of a double nerve cord situated ventrally and punctuated by segmental ganglia; from these arise the peripheral nerves, which carry sensory neurons originating mainly from sensilla on the external cuticle, and motor neurons innervating the numerous separate striated muscles. The axon of the individual neuron, measuring up to 10 microns in diameter, is enclosed in a very thin sheath only 10 millimicrons thick, which is apparently double but not myelinated (Figure 1). The axons are bundled into nerves and surrounded by dovetailed layers of neuroglial cells, and the whole is enclosed by a neural lamella, up to 8 microns in thickness, secreted by underlying perineurim cells. The ventral nerve cord, in addition, is loosely enveloped by a membrane of connective tissue. The ganglia with their neuropile of neural cell-bodies and dendrites are similarly sheathed in glial cells and lamellae. The connectives of the nerve cord contain a number of giant axons, 50 microns in diameter, running from the last abdominal ganglion to the thoracic ganglia (19).

The axon sheath consists of lipoprotein, the micelles of lipoid and protein oriented radially, while the micelles of protein within the axon are longitudinally disposed. The enclosing neural lamella is composed of protein, with radially-oriented micelles. The lipoid of the insect nervous system consist mainly of cephalins, with some lecithin and a little sphingomyelin and cholesterol; cerebrosides and neutral fat are virtually lacking in these non-myelinated nerves (18).

The resting neuron is polarized to carry an internal potential of 50–70 millivolts negative to the exterior. As in vertebrates, so in insects this resting potential derives from positive sodium ions being pumped out of the sheath, while potassium ions, to which it is freely permeable, are retained to constitute a Donnan equilibrium. The sodium pump and resting potential are maintained by oxidative metabolism. Upon stimulation, insect nerve exhibits a spike potential as entry of sodium ions causes depolarization, and there is even a slight overshoot to make the axon interior slightly positive; the spike terminates as an extra-rapid movement of potassium ions outwards re-establishes the polarization. The spike potentials complete themselves so rapidly that it is possible to have successive spikes at intervals of one millisecond. In insects these action potentials propagate at 2–3 m./sec.

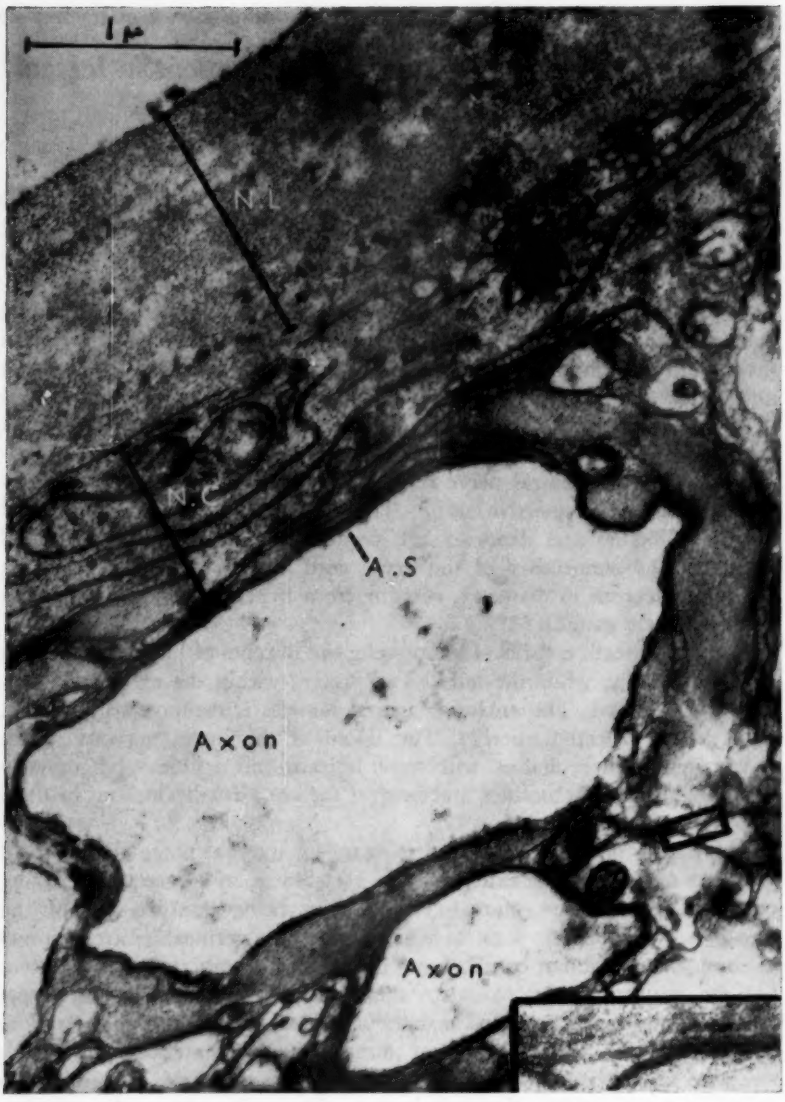

FIGURE 1. Cross-section of leg nerve 5 of *Periplaneta americana*. N.L., neural lamella; N.C., neuroglial cells; A. S., axon sheath. Insert: axon sheath × 147,000, showing double membrane. Electromicrograph by C. L. Hannay (from Colhoun, E. H. [1960]. Agric. Food Chem., *8*: 252–7).

in peripheral nerves, 6–7 m./sec. in giant fibres, which is considerably slower than the 100 m./sec. speed in the myelinated axons of vertebrates.

The synapses of an insect such as the cockroach are best studied in the last abdominal ganglion, where the sensory neurons of the peripheral cercal nerve synapse with the giant fibres. In insects the sensory neurons may fuse among themselves, so that a sensory nerve can gather inputs from many sensilla. The synaptic delay in insects is between 0.5 and 2 millisec. as in vertebrates. The chemical mediator is considered to be acetylcholine, which has been identified in insect brain in concentrations much higher than in vertebrates; moreover, insect ganglia are exceptionally rich in the hydrolyzing enzyme cholinesterase (22). Acetylcholine is without effect when injected into the haemolymph of insects, but when the neural lamella has been removed from the ganglia this compound causes continuous postsynaptic discharge. Thus the insect central nervous system resembles the mammalian autonomic system with respect to the agent for trans-synaptic conduction (29). But there are certain anomalies, which suggest that the mediator in insects may not be ACh itself, but a similar compound susceptible to hydrolysis by ChE (30).

The motor neurons terminate in a diffuse arborization with multiple end-plates, which are necessary because insect muscle does not propagate impulses well (20). The muscle that causes the locust to jump, the extensor tibialis, is innervated by a single fast fibre in leg nerve 5 with multiple end-plates; the stimulus for walking, on the contrary, is provided by a single fibre in nerve 3b with a few end-plates on the same muscle. The mediator at the neuromuscular junction is not acetylcholine, and thus curare has no effect on insects; the nature of this mediator is still being sought.

Modern insecticides are nearly all poisons of the insect nervous system. They are typically apolar compounds, to dissolve in the lipoids of cuticle and nerve sheath (14), thus overcoming the barriers to sites of action in the insect. Most of these insecticides are stable and non-volatile, so that they have a persistent residual killing effect on insects resting or walking on their deposits.

DDT is the classical residual insecticide. Pharmacologically it is a convulsant, causing ataxia, tremors, and spasms, and finally a tetanic paralysis. As it is taken into the cuticle and sensilla, it poisons the sensory neurons (21), presumably entering the lipoid axon sheath and disrupting its structure. The symmetrical umbrella shape of its molecule, anchored by chlorine atoms, is probably responsible for its deadly grip on the axons (28). The affected sensory neurons then bombard the synapses with trains of repetitive discharge, with the result that the motor neurons excite the muscle tremors known as DDT jitters. An explanation for this instability that DDT induces in sensory neurons, which resembles the effects of low external calcium, has recently been obtained from studies of the potentials within the axons (32). In DDT-poisoned insects the axon does not immediately repolarize completely, but shows a prolonged negative after-potential, and

it is in this condition that it is liable to show repetitive discharge even from its own spontaneous activity. It is thus considered that DDT has the effect of inhibiting the extra-rapid permeability to potassium necessary for immediate repolarization, and indeed it has been found that a DDT-treated nerve is unusually resistant to electrification by a forcing current.

Gamma-BHC and dieldrin are even more powerful insecticides than DDT. Both of these chlorinated hydrocarbons appear to act on the ganglion rather than the axon, because the poisoned insects show prolonged post-synaptic discharges in the giant fibres from normal cercal-nerve input; these insecticides, however, do not inhibit cholinesterase. Gamma-BHC is rapid in action, causing hyperexcitation to precede ataxia and convulsions; whereas the action of dieldrin is slow, the insect gradually developing ataxia, convulsions, and a flaccid paralysis (31).

The most recent group of insecticides, of which perhaps parathion, malathion, and Diazinon are the best known, are the organophosphorus compounds. Most of them are esters of phosphorothioic acid or of phosphorodithioic acid, containing a P=S linkage to reduce toxic hazard to mammals. This is usually immediately oxidized in the insect to the P=O linkage, producing compounds like paraoxon, malaoxon, or diazoxon, which are very potent inhibitors of cholinesterase. Their point of attack is the ganglion, so that a single presynaptic spike is multiplied into continuous post-synaptic discharge, followed eventually by synaptic block. Insects poisoned by OP compounds die with 95 per cent of their ChE inhibited; they also show much free ACh, but no more than in DDT-poisoned insects. The insecticidal effect of OP compounds and their immediate toxic derivatives is proportional to their anti-ChE activity *in vitro*. These OP compounds phosphorylate the molecule of cholinesterase at the esteratic site which normally looks after acetylcholine (14).

A new group of insecticides, the carbamates, of which Sevin and Pyrolan are quite well-known examples, are also anticholinesterases. Here again it is found that those carbamates which are least polar make the best insecticides.

It is by no means certain that the changes in nervous activity described above are sufficient to account for the death of the poisoned insects. DDT-treated insects die long before their reserves of glycogen and fat have fallen to the level where they would die of starvation. A clue to the possible ultimate cause of death is suggested by the discovery of a toxin liberated by the nerve cord of DDT-poisoned cockroaches (23; 24). This water-soluble polar material, which is not adrenalin, histamine, or other known hormonal compounds, is nevertheless probably a primary aromatic amine or its ester. Separated by chromatography, its effect on nerve is to cause a continuous increased activity and finally block. This neuroactive substance is released into the blood as the insect develops symptoms of poisoning not only from DDT, but also from the organophosphorus compound TEPP, and even mere electrical stimulation. Several other neuroactive

substances have been found by chromatography and bioassay of the blood of poisoned cockroaches; certain of these such as catechol amines are released from the neuro-secretory gland known as the corpus cardiacum (7).

Unlike mammals, which have weak points such as respiratory failure that form a fulcrum between life and death, insects die very slowly. During this twilight period there is time for the poison to be detoxified and the insect to recover, the paralysis or knockdown thus being reversible. This recovery may often be ensured by removing the poisoned insects to a higher temperature, as can be done for DDT or pyrethrin poisoning. Some species of insects, for example, the Mexican bean beetle and the tobacco hornworm, detoxify so fast that they are not poisoned with DDT in the first place.

Most species of insects are highly susceptible to these nerve poisons, but resistant populations are liable to develop as a result of Darwinian selection when these insecticides are applied extensively over a period of years. For example, populations of the housefly usually become DDT-resistant after two or three years of DDT application, and substitution of BHC or dieldrin generally results in resistance to them in the following year. In Canada, twelve species of insect and mite pests have developed resistant strains; in the world, some 140 species of arthropods have now produced resistant populations (5). Of these, fifty-six have developed DDT-resistance, seventy-eight dieldrin-resistance, and twenty-nine organophosphorus-resistance.

The physiological mechanism which distinguishes the DDT-resistant from the normal fly has been sought intensively. It does not lie in the rate of penetration of DDT through the cuticle, or in the relative content of cellular-oxidation enzymes such as cytochrome oxidase, or in other enzymes such as cholinesterase or phosphatase, or in cell constituents such as copper, glutathione, or lipoid; any interstrain differences observed proved to be related to the fly's geographical origin rather than its DDT-resistance. So far only one characteristic has been found to be correlated with DDT-resistance in the housefly, and that is its ability to detoxify DDT to the inactive compound DDE.

The enzyme responsible, which has been called DDT-dehydrochlorinase, requires glutathione as an activator (26). It also dehydrochlorinates methoxychlor and DDD, and indeed DDT-restistance extends to these related insecticides. It is competitively inhibited by other dichlorodiphenyl compounds, such as DMC, which thus act as synergists for DDT against DDT-resistant flies. The enzyme is abundant in nervous tissue, and this could explain the observed fact that the resistance is also shown by the nerves themselves. DDT-resistance and DDT-dehydrochlorinase content are inherited in parallel as if due to a single mutant gene (10). This peculiar enzyme has been purified 50,000 times; its properties most closely resemble the nitroglycerin reductase of pig liver. DDT-dehydrochlorinase has been demonstrated in species normally tolerant of DDT, such as the Mexican bean beetle and the tobacco hornworm. Perhaps the mutant allele allows the enzyme already there to become active to detoxify the substrate DDT.

DDT-resistance has been proved to be due to a single allele in *Drosophila melanogaster*, and this gene has been located at map-distance 66 on chromosome 2; however, in this species the metabolite is not DDE but Kelthane (Figure 2). The detoxification process, involving not dehydrochlorination but oxidation of the ethane nucleus of DDT, also takes place in the American cockroach, where it is effected by a mixed-function oxidase activated by

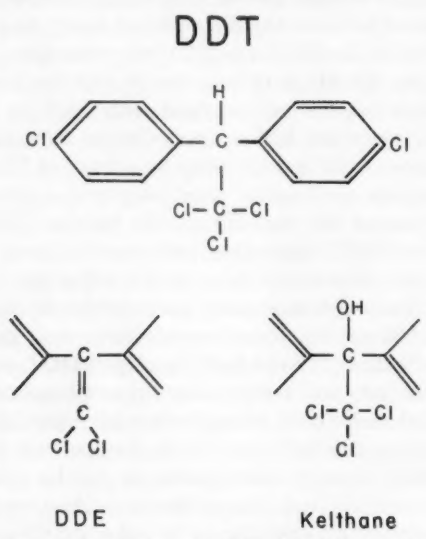

FIGURE 2. Routes of DDT detoxification to DDE or Kelthane.

TPNH, nicotinamide, and Mg ions. TPNH also activates glutathione reductase; evidently in some species of insects the oxidase system takes this electron donor and Kelthane is produced, whereas in others the reductase takes it and glutathione is produced, to activate DDT-dehydrochlorinase to produce DDE (2). In the body louse, DDT-resistant strains detoxify DDT *in vivo* to a conjugate of a metabolite that is possibly p-chlorobenzoic acid, by an enzyme that is heat-stable and which is also present but inoperative in susceptible strains (17).

DDT-resistance is an important public-health problem, particularly in mosquitoes that transmit malaria and other diseases. Dehydrochlorination to DDE has been found to occur in DDT-resistant *Anopheles* and *Culex* (16). At the University of Western Ontario we have been making a special study of DDT-resistance in the yellow-fever mosquito, *Aedes aegypti*. We have found that the DDT-resistant strains produce DDE but not Kelthane, but so far have been unable to find the conditions to demonstrate the enzyme DDT-dehydrochlorinase *in vitro*. DDT-resistant strains of Carib-

bean origin produce much DDE whereas their susceptible counterparts produce very little DDE. A normal population of Malayan origin, already slightly DDT-tolerant, produces a considerable amount of DDE, whereas a DDT-resistant strain developed from it produces little more DDE (6).

The Caribbean DDT-resistant strains were found to have an additional defence mechanism (Figure 3); their larvae when placed in DDT suspensions are stimulated to excrete long tubular lengths of peritrophic membranes, carrying within them the orally-ingested DDT which has thus

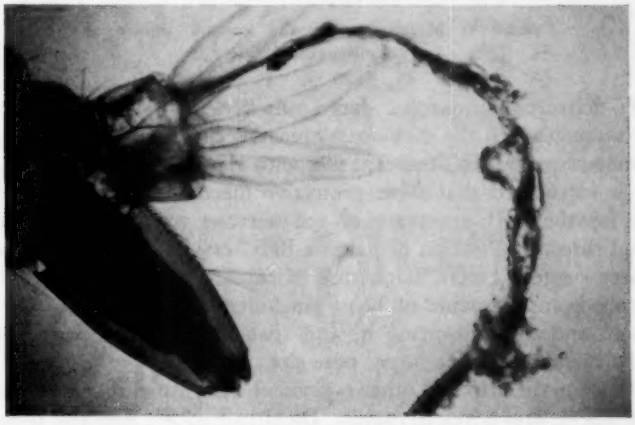

FIGURE 3. Excretion of peritrophic membrane by larvae of DDT-resistant *Aedes aegypti*.

not been absorbed into the body. The Malayan DDT-resistant strain does not show this property (1). On the other hand, the Malayan resistant strain shows an increased content of phospholipid, which the Caribbean resistant strains do not, and absorbs DDT much less than the normal (8). A third Malayan strain made highly cross-resistant to DDT by selection pressure with malathion also showed higher phospholipid, and absorbed DDT and malathion very much less than the normal. Thus it may be seen that strains of the same species from widely-separated geographic origins can develop DDT-resistance by different physiological mechanisms.

Resistance to gamma-BHC and dieldrin are evidently one and the same entity, since BHC-resistant insects are found to be also dieldrin-resistant, and *vice versa*; but there is no cross-resistance to DDT. Indeed the actions of gamma-BHC and dieldrin on the ganglia are similar, and the spatial configuration of the chlorine atoms in the gamma-BHC molecule is similar to that in dieldrin and the other related cyclodiene derivatives. BHC-resistant strains of houseflies absorb gamma-BHC less and detoxify it more than normal strains (Figure 4). The first detoxification product to be identified was pentachlorocyclohexene (25), although it is not produced

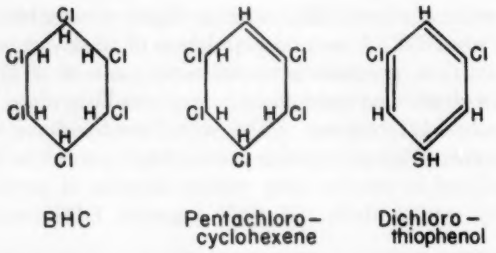

FIGURE 4. Metabolites of the gamma isomer of benzene hexachloride.

by DDT-dehydrochlorinase; a large number of water-soluble products appear, among which the dichlorothiophenols can be produced by incubating homogenates of BHC-resistant flies with glutathione *in vitro* (3). However, it is considered that these protective mechanisms are insufficient to account for the BHC-resistance of the nervous system itself, which can withstand direct application of gamma-BHC crystals to the ganglion (4). It has been suggested from biophysical calculations (12) that gamma-BHC is the only isomer capable of being anchored in the pores of the nerve membrane and thus distorting it, and that resistant strains are those in which the membrane has a larger pore size.

Resistance to dieldrin and other cyclodiene derivatives is characteristically strong, decisive, and develops rapidly. Dieldrin-resistant flies and mosquitoes absorb dieldrin no less than normal, and there is no detoxication either in the resistant or in the susceptible strains. All that seems to happen to these cyclodiene derivatives in the insect tissues is that they are epoxidized —heptachlor to heptachlor epoxide, aldrin to dieldrin, and isodrin to endrin (Figure 5)—the corresponding epoxides being about as toxic as their precursors. Resistance to these cyclodiene derivatives may be associated with the fat-body, since dieldrin and heptachlor epoxide are known to accumulate in that organ in roaches and flies; while normal mosquitoes may be made more dieldrin-tolerant by providing nourishment to develop their fat-body. The inheritance of dieldrin-resistance has given clear-cut results, since the character usually segregates sharply; a single gene allele has been found responsible for dieldrin-resistance in the African malaria mosquito and the Australian sheep blowfly. In *Aedes aegypti*, we have found that the dieldrin-resistance of a strain from Puerto Rico segregates monofactorially; but unlike the classical type of dieldrin-resistance, it carries a strong cross-resistance to DDT. The genetic factor proved to show linkage with the mutant allele *yellow* on Chromosome 2, at a cross-over distance of approximately 25 (9). The DDT-resistance in the Caribbean and the Malayan strains mentioned previously also showed linkage with Chromosome 2.

Resistance to organophosphorus compounds, introduced as remedial insecticides for chlorinated-hydrocarbon resistance, has now developed in

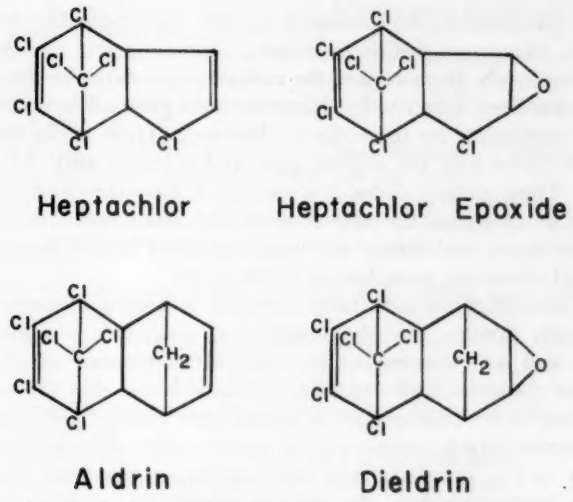

FIGURE 5. Cyclodiene derivatives and their epoxides.

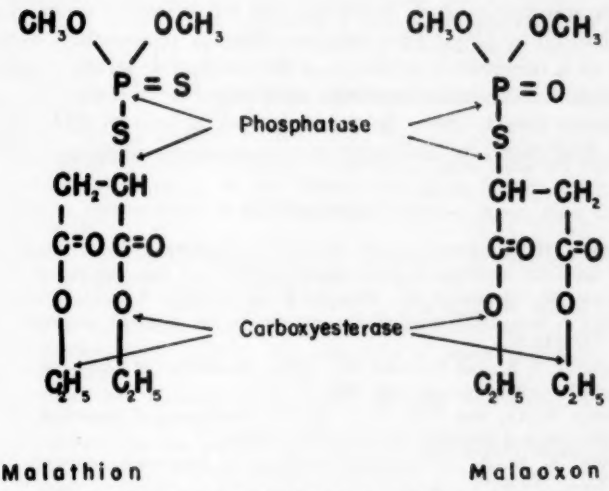

FIGURE 6. Points of enzymic hydrolysis of malathion and malaoxon.

seventeen species of insects and twelve species of plant-feeding mites. In the housefly, malathion-resistance has been found to be a different entity from parathion-resistance. However, both the resistances result from the increased detoxication of malaoxon and paraoxon respectively by particular phosphatase enzymes (Figure 6). Concomitant with the increase in a

particular phosphatase, the resistant flies show a corresponding reduction in aliesterase, an enzyme which hydrolyses aliphatic esters and is inhibited by OP compounds. In each case, the malathion-resistance or the parathion-resistance has been found to be due to a single gene allele which is simultaneously responsible for the reduced aliesterase. These alleles form a multiple allelic series with the normal gene and a fourth allele for Diazinon-resistance. These mutant alleles, it is concluded, have the effect of modifying the active groupings of the aliesterase so that the enzyme is now rapidly dephosphorylated, and thus is no longer inhibited by OP compounds but becomes a hydrolysing phosphatase for them (15).

In our laboratory we have been fortunate in having available for study an absolutely specific malathion-resistance, restricted to malathion and malaoxon and not extending to any other OP compound or other insecticide, in the mosquito *Culex tarsalis*. We have found that the resistance is accompanied by an increase, not in phosphatase activity, but in the enzyme carboxyesterase which hydrolyses the succinic-ester side-chain peculiar to malathion (11). This increased carboxyesterase has been found to be genetically inseparable from the malathion-resistance, which is inherited as if it were due to a single partially-dominant gene allele. The Michaelis constant of carboxyesterase activity in the resistant strain is identical with that in the susceptible strain, indicating that the difference is one of quantity rather than of quality. The enzyme–substrate relationship suggests the presence of a competitive inhibitor in the susceptible strain, implying that the function of the mutant resistance allele might be to remove the inhibitor of an enzyme already there. Be that as it may, at least in *Culex tarsalis* we have the third case to be discovered of a gene-enzyme-resistance relationship.

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